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GERMINATION AND GROWTH INHIBITORS OF ALFALFA

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Alfalfa (*Medicago sativa* L., Leguminosae) is a legume of considerable economic importance in the United States. Problems associated with the continuous growing of alfalfa were known more than 80 years ago (1,2). Many studies conducted during the past 20 years have shown the autotoxicity of alfalfa (1–7), but few investigations have identified any compound(s) responsible for the effect (8,9). Autotoxicity is expressed in yield reduction and difficulty of reestablishment in fields where alfalfa was previously grown (8). Saponins, although implicated in toxicity, have been dismissed as autotoxic agents in alfalfa (8).

Our research objective was to isolate and identify the compound(s) causing autotoxicity. We examined alfalfa foliage because, presumably, it has the greatest phytotoxic effect (4). Lettuce seed germination was chosen as our bioassay because it is sensitive, easy, and generally used in routine screening (10). In some of the early bioassays curly cress seed (11) replaced lettuce seed, and later, alfalfa seed was also used when testing pure compounds. Root lengths of both sample and control seedlings were recorded whenever sample seedlings differed visually from the control seedlings.

Cc of EtOAc solubles (obtained by solvent partition) produced 14 fractions, 6 of which completely inhibited germination at the 10 mg per dish level. The

first of these six active fractions was purified by preparative tlc to yield 30 mg of a "single component" (by tlc), but gc-ms revealed at least eight components: several unidentified compounds, medicarpin [1], sativan [3] and the methoxy analogues of the last two named compounds. These compounds are often produced as phytoalexins when legumes are
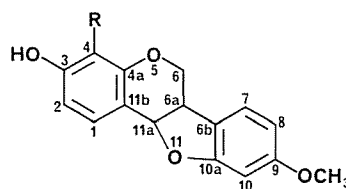
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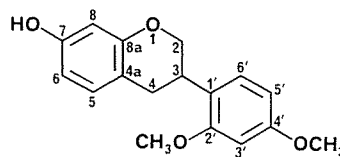
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infected, diseased, or otherwise stressed (12). Pure medicarpin [1] for the bioassays was obtained from alfalfa roots, a known source of medicocarpin [the 3-*O*-glucoside of medicarpin (13)]. The methoxymedicarpin was isolated in sufficient quantity and purity from one of the other active column fractions of the EtOAc solubles. It was identified as 4-methoxymedicarpin [2] by ms and nmr. Both of these pterocarpans, originally isolated from the heartwood of a tropical tree (14,15), were active in our bioassay (Table 1). These compounds are apparent contributors to autotoxicity of alfalfa as medicarpin [1] inhibits germi-

TABLE 1.   Assay Data.

Sample	Concentration molar	Germination[a] Sample (Control)		Seedling Growth Mean Root Length Sample (Control)[b]	
		Lettuce number	Alfalfa number	Lettuce cm	Alfalfa cm
Medicarpin [1]	1×10^{-3}	$18^c(31)$	$13^d(32)$	$0.8^d(1.9)$	—
4-Methoxymedicarpin [2] . . .	1.7×10^{-3}	—	39 (40)	—	$1.8^d(4.0)$
	0.8×10^{-3}	$6^d(35)$	—	—	—

[a]40 seeds per treatment or control.

[b]Standard deviations of root lengths are 0.98 cm (lettuce) and 1.2 cm (alfalfa), calculated from variance pooled over the treatment and control.

[c]Significantly different from controls at $p \leq 0.01$ by Chi-square 1-tail test.

[d]Significantly different from controls at $p \leq 0.001$ by Chi-square 1-tail test (germination) or Student's *t* test (seedling growth).

nation of alfalfa seeds (59% after 6 days at 10^{-1} M, $p \ll 0.001$), and 4-methoxymedicarpin [2] inhibits growth of alfalfa seedlings (55% after 5 days at 1.7×10^{-3} M, $p \ll 0.001$). We are aware of only one previous report of a pterocarpan inhibiting seed germination; it is associated with "clover sickness" (16, 17).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H- and ^{13}C-nmr spectra were obtained at 300 and 75.47 MHz, respectively, with CDCl$_3$ as the solvent. Ms were obtained at 70 eV in the electron impact mode. Both gc and gc-ms were obtained with 15 m DB-1 column, temperature programmed at 4°/min from 100° to 300°. A 6-cm (i.d.) × 62-cm glass column, slurry packed with 450–500 g 60–200 mesh Si gel (J.T. Baker), was used for cc. Commercial 20 × 20 × 0.25 cm precoated Si gel 60 F$_{254}$ (Merck) and 20 × 20 taper plates (Analtech) were used for tlc. A 10 × 500 mm C$_{18}$ ODS column (Whatman) and a refractive index detector (Waters) were used for hplc.

PLANT MATERIAL.—Dried alfalfa (*M. sativa* 'Moapa') foliage and roots were obtained from greenhouse and field plantings at Michigan State University, East Lansing, Michigan. This is a commercially available alfalfa variety; no voucher specimen is available.

BIOASSAY.—Surface-sterilized lettuce (*Lacabatuca sativa*) and alfalfa ('Vernal') seeds were sown on treated filter paper in petri dishes, 20 per dish and 2 dishes per treatment; 4 ml H$_2$O was added, and the dishes were covered and wrapped in Al foil (18). Treatments varied from 40 mg (crude extract) to 1 mg (pure compound) per dish. Seeds were incubated for 5 days uniformly; after-

wards the number of germinated seeds was recorded. For every two treatments, a blank control was added. The treatment and associated control were run at the same time (on the same day). Seedling growth was measured by recording root lengths of both control and treated seedlings.

EXTRACTION AND ISOLATION.—Ground foliage (3.8 kg) was extracted sequentially via percolation with pentane/hexane and 95% EtOH. The EtOH solids (281 g) were dissolved in MeOH-H$_2$O (1:1) and extracted with hexane. The aqueous phase was adjusted to H$_2$O-MeOH (3:1) and extracted with EtOAc. The EtOAc solubles (15 g) were then chromatographed on a silica column by elution first with 3 liters EtOAc-hexane (1:4), followed by 2.5 liters EtOAc, and finally by 2.5 liters MeOH. Fourteen 500-ml fractions were collected, six of which completely inhibited germination of lettuce seeds. One of these active column fractions (72 mg) was separated by preparative tlc with EtOAc as the developing solvent; the area about R_f 0.6 (30 mg) was nearly all medicarpin (gc, ms, ^{1}H, and ^{13}C nmr), but attempts to purify it further were unsuccessful. Pure medicarpin [1] was obtained from alfalfa roots (13). A second active column fraction (800 mg) was separated by hplc into 5 fractions on a reversed-phase column by eluting with MeCN-H$_2$O (3:2). Hplc fraction 4 (260 mg) was separated further by tlc (Si gel G taperplate and CH$_2$Cl$_2$-MeOH, 94:6). The fastest moving band was scraped from the plate, extracted from the Si gel with 20% MeOH in CH$_2$Cl$_2$, the solvent removed, and the sample weighed. The sample (1 mg sample per 10 μl 20% MeOH in CH$_2$Cl$_2$) was streaked on another plate (20 × 20 × 0.25 cm precoated commercial plate), developed with 6% MeOH in CH$_2$Cl$_2$, and the process repeated until only one peak was detected by gc for the recovered sample. In this manner we

obtained 16 mg of 4-methoxymedicarpin [2] for nmr, ms, and bioassays. Separations were not optimized for highest yield.

Tlc on Si gel 60 F_{254} with 4% MeOH in CH_2Cl_2 gave R_f s of 0.6 and 0.8 for medicarpin and 4-methoxymedicarpin, respectively.

COMPOUND IDENTIFICATION.—Preliminary structural identification of isolated compounds was established by gc-ms, and confirmatory evidence was afforded by ^1H and ^{13}C nmr. The mass spectra of sativan [3], medicarpin [1], and 4-methoxymedicarpin [2] matched literature values (19,20,21, respectively). ^1H and ^{13}C nmr of medicarpin resembled literature values (22,23). ^1H nmr of the acetate of 4-methoxymedicarpin is given in the literature (24), but we report the nmr for the free compound. 4-Methoxymedicarpin [2]: ^1H nmr (300 MHz, $CDCl_3$) δ 3.58 (ddd, J = 10.8, 6.6, 4.6, H-6a), 3.65 (dd, J = 10.8, 10.5, H-6ax), 3.75 (s, OMe at C-9), 3.90 (s, OMe at C-4), 4.34 (dd, J = 10.5, 4.6, H-6eq), 5.53 (d, J = 6.6, H-11a), 6.45 (d, J = 2.3, H-10), 6.45 (dd, J = 8.9, 2.3, H-8), 6.66 (d, J = 8.6, H-2), 7.05 (d, J = 8.6, H-1), 7.13 (d, J = 8.9, H-7); ^{13}C nmr (75.47 MHz, $CDCl_3$) δ 39.6 (C-6a), 5.55 (OMe), 56.3 (OMe), 66.9 (C-6), 78.4 (C-11a), 96.9 (C-10), 105.4 (C-8), 106.4 (C-2), 114.0 (C-11b), 118.8 (C-6b), 121.1 (C-1), 124.7 (C-7), 134.0 (C-4), 143.2 (C-3), 147.3 (C-4a), 160.6 (C-10a), 161.1 (C-9).

ACKNOWLEDGMENTS

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